SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Comparative analysis of NLRP3 inflammasome-mediated IL-1β production in different types of human monocyte-derived macrophages

by

Marietta Margit Budai

Supervisor: **Szilvia Benkő**, PhD

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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Supervisor: Dr. Szilvia Benkő, PhD

Doctoral School of Molecular Cell and Immune Biology
University of Debrecen

Head of the **Examination Committee**: Prof. Dr. László Fésüs, MD, PhD, DSc, MHAS
Members of the Examination Committee:
Prof. Dr. Zsuzsanna Bajtay, PhD, DSc
Prof. Dr. Andrea Szegedi, MD, PhD, DSc

The Examination takes place at the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen at 11 AM , 20th of June, 2017.

Head of the **Defense Committee**: Prof. Dr. László Fésüs, MD, PhD, DSc, MHAS
Reviewers: Prof. Dr. Péter Nagy, MD, PhD, DSc
Dr. Ádám Dénes, MD, PhD
Members of the Defense Committee:
Prof. Dr. Zsuzsanna Bajtay, PhD, DSc
Prof. Dr. Andrea Szegedi, MD, PhD, DSc

The PhD Defense takes place at the Lecture Hall of the "A" Building, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 1 PM, 20th of June, 2017.

Introduction

IL-1β is a "master" cytokine regulating a wide variety of physiological and immunological processes. Due to its potent ability, the secretion of the active form of the cytokine is under tight regulation, mediated by multiprotein complexes called inflammasomes. One of the most studied inflammasome complexes is the NLRP3 inflammasome that contains multiple copies of NLRP3 sensor, ASC adaptor, and caspase-1 enzyme. It has been proposed that activation of the NLRP3 inflammasome requires two distinct signals. The first, so called priming signal, is typically induced by Toll-like receptors (TLRs) that triggers the expression of pro-IL-1β and components of the inflammasome (NLRP3), through several signal transduction pathways. The second signal leads to the formation of inflammasome complex, the activation of caspase-1, and the cleavage of inactive pro-IL-1β into a mature cytokine. The second signal may be triggered by a wide variety of molecules that function as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs).

Human macrophages; the most frequently studied models for NLRP3 inflammasome functions, can release IL-1β only when both of these events occur in parallel. Macrophages are very plastic, and may be characterized by different morphological and functional properties. Homeostatic control of human monocyte/macrophage development is mostly dependent on their microenvironment, and influenced by macrophage colony stimulating factor (M-CSF) or granulocyte-macrophage colony stimulating factor (GM-CSF). Macrophages are potent inducers and regulators of the immune response. Apart from the different phases of an inflammatory response to infections or skin damage, macrophages take a central role in orchestration of the resolution of inflammation, wound healing, and also in new tissue formation and tissue remodeling.

Here, we report a comparative study focusing on the dynamics and molecular mechanisms of NLRP3 inflammasome priming and activation in LPS-stimulated human blood monocyte-derived GM- or M-macrophages, differentiated in the presence of GM-CSF or M-CSF, respectively. Furthermore, we aimed to study the effect of the immunomodulatory natural compound *Aloe Vera*, which has been used in traditional medicine related to infections and inflammation, as well as wound healing in our system.

2. Theoretical background

2.1. Danger- and pathogen-recognition system

The innate immune cells provide the first line of defense against pathogenic organisms through the recognition of different pathogen-associated molecular patterns (PAMPs) by their pattern-recognition receptors (PRRs). However, even self-derived molecules or noninfectious foreign substances called danger- (or damage-) associated molecular patterns (DAMPs) can elicit immune responses by stimulating the innate immune cells. Activation of the PRRs ultimately leads to the production of cytokines that drive the pathogen-derived or sterile inflammatory responses.

2.2. Nod-like receptors

It is generally characteristic for NLR proteins to contain an N-terminal protein-protein interaction domain required for oligomerization, a central NACHT domain (or NBD - nucleotide-binding domain) necessary for self-oligomerization and activation, and a C-terminal leucine-rich repeat (LRR) that confers ligand recognition. NLR proteins recognize a specific repertoire of PAMP or DAMP molecules. In humans, there are 22 known NLRs that are divided into five subfamilies according to the type of their N-terminal domain: NLRA, NLRB, NLRC, NLRX and NLRP. Based on their primary or best-characterized functions, we can divide the receptor family into three subgroups: Members of the most characterized subgroup are able to form inflammasome complexes that have indispensable role in the maturation of IL-1β and IL-18 pro-inflammatory cytokines. The function of another subgroup is associated with positive or negative regulation of inflammatory signaling cascades. Interestingly, it seems that the third subgroup has an important role in reproduction and embryonic development, which indicates that the function of NLRs is not restricted only to immune functions**.**

2.2.1. Inflammasomes

Inflammasomes were described in 2002 by the research group of Tschopp as multiprotein complexes containing NLR sensor, ASC adaptor and caspase-1 enzyme. While inflammasome complexes are able to sense a broad range of activators, these multiprotein complexes share a common effector function. Upon stimulation, they oligomerize and

caspase-1 enzyme becomes activated, which subsequently leads to the cleavage of the inactive pro-IL-1β and pro-IL18 to their matured IL-1β and IL-18 forms for secretion.

Caspase-1 is synthesized as a catalytically weak precursor; pro-caspase-1, that is activated through auto-proteolysis releasing its large (p20) and small (p10) subunits. This way of inflammasome activation has been termed the canonical pathway. Besides canonical IL-1β production, recently, an alternative (non-canonical) way of inflammasome activation has been described, which promotes the activation of caspase-11 (in mice) or caspase-4 and caspase-5 (in human).

Due to its diverse and indispensable functions in inflammatory- and immune responses, IL-1β is considered as a master regulatory cytokine. For example, IL-1β induces the production of other inflammatory cytokines and chemokines, it also enhances the expression of adhesion molecules on endothelial cells which trigger infiltration of inflammatory and immune cells. In addition, IL-1β enhances pain sensitivity, causes fever and vasodilation, thereby orchestrating local and systemic inflammatory responses underlying a broad spectrum of diseases.

2.3. NLRP3 inflammasome

NLRP3 was first described in 2002 as PYPAF1 or Cryopyrin. Today, NLRP3 is the most studied member of the NLR family. The uncontrolled NLRP3 inflammasome activity may cause serious inflammatory diseases. One group of disorders caused by the malfunction of NLRP3 inflammasome is Cryopyrinopathies. These auto-inflammatory syndromes are characterized by recurrent systemic inflammation that involves several tissues including joints and skin. NLRP3 inflammasome activation is also a key feature in the pathogenesis of protein misfolding diseases (e.g. Prion disease, Alzheimer's disease, Parkinson's disease), metabolic diseases (e.g. Type 2 diabetes), in occupational diseases (silicosis and asbestosis) and also in different allergic responses.

Due to the potent role of IL-1β the activation of NLRP3 inflammasome is tightly controlled by a two-step process, at both transcriptional and post-transcriptional level. The first so called "priming" signal is typically induced by diverse pathogen-related molecules through specific receptors such as Toll-like receptors. This event results in the transcriptional upregulation of pro-IL-1β and the inflammasome components through diverse signal transduction pathways like those involving p38, ERK and NF-κB. The second signal leads to the formation of inflammasome complexes, which initiates a proteolytic cleavage of caspase-

1 and mediates the maturation of pro-IL-1β into active cytokine. This signal can be triggered by many microbial (PAMPs) or non-microbial (DAMPs) molecules. Today it is generally accepted that NLRP3 inflammasome-mediated IL-1β secretion is not always associated with the presence of an infectious agent, it occurs upon 'sterile' inflammation as well.

Considering the broad spectrum of NLRP3 activators, it is unlikely that all of these different stimuli are sensed directly. Instead, it is generally believed that all of these signals converge to a common molecular event that eventually activates the complex. Several cellular mechanisms for NLRP3 activation have been proposed, such as sensing of K^+ ion efflux due to microbial toxins or ATP; mtROS or mtDNA due to mitochondrial destabilization or sensing of cathepsins due to lysosomal instability.

2.3.1. Potassium efflux-mediated NLRP3 inflammasome activation

One of the main signals for NLRP3 inflammasome activation is the intracellular potassium level which is crucial for the activation of caspase-1. Potassium efflux may be achieved either by microbial toxins such as nigericin acting as potassium ionophores, or by the opening of potassium channels as a response to increased extracellular ATP concentration. Tissue damage and inflammation may lead to the release of high doses of ATP that act as danger signal through the purinergic P2X7 receptor initiating the activation of the NLRP3 inflammasome. Extracellular concentration of ATP is controlled by ectonucleotidases such as CD39 that hydrolyzes ATP to AMP and CD73 that converts AMP to adenosine. Adenosine that regulates many physiological processes may be recognized by four adenosine receptor subtypes; A1, A2a, A2b and A3.

2.3.2. ROS as activators of NLRP3 inflammasome

Mitochondria constitute the biggest source of cellular ROS, and NLRP3 has been reported to associate with the mitochondria upon activation suggesting that NLRP3 might sense these products. Following studies also supported the fact that scavenging of mtROS efficiently suppresses activation of the NLRP3 inflammasome. Furthermore the oxidative stress mediator thioredoxin-interacting protein (TXNIP) associated with NLRP3, leading to inflammasome activation upon increase of cellular ROS.

2.3.3. Mitochondrial dysfunction as activator of NLRP3 inflammasome

In addition to mtROS, oxidized mitochondrial DNA (mtDNA) released into cytoplasm from the damaged mitochondria has also been reported to directly induce NLRP3 inflammasome activation. Recent studies show that accumulation of damaged mitochondria and excessive release of mtDNA in the cytoplasm due to impaired autophagy can lead to hyperactivation of the NLRP3 inflammasome.

2.3.4. Lysosomal destabilization and increased icCa^{2+} level as activators of NLRP3 inflammasome

Sterile particulate and crystalline; including monosodium urate crystals (MSU), alum, silica, amyloid-β as well as infectious bacteria (i.e. Mycobacterium tuberculosis) are phagocytosed by antigen presenting cells. This mechanism results in lysosomal rupture and the cytoplasmic release of lysosomal proteases, including cathepsin B, which has been reported to be involved in NLRP3 activation. Notably, lysosomal destabilization is sufficient to trigger Ca^{2+} mobilization, and Ca^{2+} was suggested as a key molecular regulator of the NLRP3 inflammasome.

2.3.5. Intracellular localization of NLRP3 inflammasome

Under resting conditions, most NLRP3 proteins were shown to associate with the endoplasmic reticulum (ER) homogeneously in the cytosol. This localization changed significantly after inflammasome stimulation, where NLRP3 located into the perinuclear space and co-localized with ER and the mitochondria-associated ER membranes (MAMs). Recently, mitochondria were shown to serve as molecular platforms for the NLRP3 inflammasome. It was reported that the mitochondrial antiviral signaling (MAVS) protein associates with NLRP3 and facilitates its oligomerization, leading to caspase-1 activation.

2.4. The lipopolysaccharide receptor, TLR4

Toll-like receptors (TLRs) have been established to play an essential role in the activation of innate immunity. The research group of Beutler has revealed that TLR4 is activated by a potent PAMP, the lipopolysaccharide (LPS), which is the major component of the outer membrane of Gram-negative bacteria. Many studies have demonstrated that LPS stimulation of human macrophages activates several intracellular signaling pathways that

include the NF-kB pathway and three MAPK pathways: ERK 1/2, JNK and p38. These signaling pathways in turn activate a variety of transcription factors, which coordinate the induction of many genes encoding inflammatory mediators.

2.5. Macrophages

Macrophages (MFs) are potent inducers and regulators of immune responses. MFs originate from hematopoetic stem cells in bone marrow, which develop into blood circulation monocytes, followed by regulated migration into different tissues, either in steady state or upon inflammation. The development and polarization of MFs is highly dependent on the specific microenvironment. MFs are very plastic and may be characterized by different morphological and functional properties. Several protocols have been developed in order to model these systems and enable the detailed study of MFs in *in vitro* conditions. In murine, MFs are purified from the peritoneum or the alveolar lavage, or differentiated from bone-marrow in the presence of M-CSF. While in human, MFs are differentiated from monocytes, and either M-CSF or GM-CSF provides the condition for their development.

2.5.1. Macrophages differentiated by M-CSF (M-MFs)

In M-MFs analysis highlighted a concerted down-regulation of pro-inflammatory cytokines, such as IL-1 and IL-8, but an opposite increase of the anti-inflammatory cytokine, IL-10 was observed in response to TLR activation. IL-10 is an inhibitor of activated MFs and DCs, and thus, involved in the control of innate immune reactions. IL-10 functions as a negative feedback regulator, because it is produced by macrophages, and simultaneously, it is able to inhibit their functions. In effort to characterize M-CSF-differentiated macrophages, several lines of evidences suggest a major role in host defense, wound healing and immune regulation.

2.5.2. Macrophages differentiated by GM-CSF (GM-MFs)

Human macrophages generated by GM-CSF are associated with induction of inflammatory properties. GM-CSF promotes these characteristics *via* induction of pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα, and chemokines, such as CCL22, CCL24, CCL5 and CCL1, which contribute to leukocyte recruitment. Furthermore, these

macrophages strongly express the costimulatory receptors CD86 and CD80 or HLA-DR underlining their role as potent antigen presenting cells.

It is important to note that in mouse, GM-CSF treatment of bone marrow cells induces the development of dendritic cells, while M-CSF-treated bone marrow cells are commonly used to generate macrophages. In contrast, in human, immature DCs are differentiated from monocytes in the presence of GM-CSF plus IL-4. At the same time, M-CSF- or GM-CSF-treated human blood monocytes are a frequently used, which comprise a convenient population to study macrophage function and signaling. Though both differentiation methods results in the generation of macrophages, M-CSF shifts the phenotype of macrophages toward an anti-inflammatory direction, whereas GM-CSF promotes a pro-inflammatory phenotype.

2.6. *Aloe vera*, an immunomodulatory natural compound

The mainstay of therapy for inflammatory diseases for many years has been synthetic, anti-inflammatory drugs, although their long-term use increases the risk of several side effects. At present, the trend towards the use of natural remedies with fewer side effects has given rise to natural compound as an alternative therapy for diseases.

Only a few species of *Aloe* have commercial importance, of which *Aloe barbadensis miller*, commonly referred to as *Aloe vera* is considered the most potent plant. *Aloe vera* has been used in traditional medicine in many cultures for over 2000 years, such as China, India and Japan. Over 75 active components have already been identified in leaf gels and many of them have been implicated as immunomodulatory compounds.

Commonly, *Aloe vera* has been used externally to treat various skin conditions such as cuts, burns and eczema. *Aloe* gel has a wound healing effect and several mechanisms have been proposed as an explanation for its activity, including maintaining wound contraction, increase epithelial cell migration, more rapid synthesis of collagen and reduction in inflammation. Studies, which examined anti-inflammatory activities of the *Aloe vera* gel, suggested that these effects occur *via* suppression of pro-inflammatory cytokines (e.g. TNFα, IL-1β, IL-6). However, polysaccharides or the anthraquinone component, emodin in *Aloe vera* gel were demonstrated as activators of macrophages and NK cells. Besides its anti-inflammatory effects, anti-microbial, anti-oxidant, anti-cancer, anti-diabetic properties of *Aloe vera* have also been shown. Moreover, *Aloe vera* is cheap, easily available and applicable with minimal equipment, properties which enable future development of multidirectional therapeutic action.

2.7. Aims of the study

Aim 1. Study the different dynamics of NLRP3 inflammasome-mediated IL-1β production in human GM-MFs and M-MFs.

Though NLRP3 is one of the most studied inflammasome complexes, there are no comparative studies focusing on the LPS-primed IL-1β secretion *via* NLRP3 inflammasome activation in human MFs differentiated by M-CSF or GM-CSF. Despite plasticity and different morphological and functional characteristics of human macrophages, previous studies have focused mainly on general gene expression differences and some specific functions (such as phagocytosis, migration, etc.) of these cells. In order to gain a better understanding of the regulation of NLRP3 inflammasome by LPS-primed human macrophages our study aimed to:

- Monitor the time-dependent pattern of cytokine expression of LPS-primed M-MFs and GM-MFs.
- Identify the involvement of NLRP3 inflammasome-dependent caspase-1 activity in LPS-induced IL-1β production by both macrophage subtypes.
- Examine the signal transduction pathways and expression pattern of inflammasome components in the two macrophage types.
- Analyze the modulatory effect of released endogenous ATP and that of extracellular adenosine on IL-1β secretion.
- Investigate the effect of robust IL-10 secretion by M-MFs on IL-1β secretion.

Aim 2. Study the effect of immunomudolatory *Aloe vera* on LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages.

The anti-oxidant, anti-bacterial and anti-inflammatory benefits of *Aloe vera* have been examined and described in several studies, nevertheless, its role on the function of human monocyte-derived MFs; especially on the production of potent pro-inflammatory cytokine IL-1β, has not been investigated. Therefore, we aimed to study the effect of *Aloe vera* on

- Cytokine secretion, especially that of IL-1β by human macrophages.
- Expression of NLRP3 inflammasome members as well as the precursor of IL-1β.
- Possible modulatory effect of *Aloe* on the activation of the LPS-induced main signaling pathways.
- Expression of ATP-sensing P2X7 receptor in LPS-primed macrophages.

3. MATERIALS AND METHODS

Reagents

Ultrapure LPS from *Escherichia coli* was purchased from InvivoGen (San Diego, CA, USA). ATP, A740003 (selective P2X7 purinoceptor antagonist), ARL67156 (ecto-ATPase inhibitor), apyrase (high activity), ZM241385 (A2a-specific antagonist), PSB1115 (A2b-specific antagonist), DPCPX (A1-specific antagonist) and VUF5574 (A3-specific antagonist) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Z-YVAD-FMK (caspase-1 inhibitor) was purchased from BioVision Technologies (Milpitas, CA, USA). Anti-human IL-10 was obtained from Thermo Fisher Scientific (San Diego, CA, USA) and recombinant human IL-10 was from PeproTech (Rocky Hill, NJ, USA).

Commercially available *Aloe vera* gel (Forever Living Products Hungary Ltd) was centrifuged with 2000 rpm for 30 min to remove the insoluble components and obtain a clear solution. Solvent utilized for the preparation of the gel was used in the mock experiments. The supernatant of the centrifugation was used in the experiments.

THP-1 cell culture and differentiation into macrophage

The human monocytic THP-1 cells (ATCC TIB-202) were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 2mM L-glutamine and supplemented with 10% heat-inactivated-FCS, 500 U/ml penicillin-streptomycin (Thermo Fisher Scientific). Differentiation of THP-1 cells was induced by incubating the cells with 0.5 μM phorbol myristate acetate (Invivogen) for 3 hours, as described previously (Grahames et al, 1999). Then cells were washed three times with PBS and plated at $1{,}3x10^6$ /ml. After 24 hours cells were treated with 100 ng/ml LPS in the absence or presence of *Aloe vera*.

Monocyte isolation and macrophage differentiation

Leukocyte-enriched buffy coats were obtained from healthy blood donors in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Ethics Committee of the University of Debrecen (Debrecen, Hungary). Informed consent of all participating subjects was obtained. Mononuclear cells were isolated by density gradient centrifugation using Ficoll Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, UK) and $CD14^+$ monocytes were separated with anti-CD14–conjugated microbeads (Miltenyi Biotec, Bergish Gladbach, Germany). Finally, monocytes were plated in 24-well tissue culture plates ($1{,}5x10^6$ cells/ml) in RPMI 1640 (Sigma-Aldrich)

containing 2 mM L-glutamine and supplemented with 10% heat-inactivated-FCS, 500 U/ml penicillin-streptomycin (Thermo Fisher Scientific) and differentiated for 5 days in the presence of either 50 ng/ml M-CSF (PeproTech) or 80 ng/ml GM-CSF (Gentaur Molecular Products, Kampenhout, Belgium). On day 2, M-CSF or GM-CSF was replenished. On day 5, macrophages (M-MFs, GM-MFs) were stimulated with LPS (500 ng/ml) for the indicated time periods. When indicated, prior to sample collection, supernatants of macrophages were replaced with FCS-free RPMI media supplemented with ATP (5 mM) for 45 minutes. In some experiments, adherent $CD14^+$ monocytes following isolation were primed with LPS.

Cytotoxicity assay

Monocytes were plated and differentiated in 96 well plates at a concentration of $2x10^5$ cells / well. On day 5, macrophages (M-MFs, GM-MFs) were incubated with LPS for the indicated time at 37^oC. At the end of treatment, the medium from control and LPS-treated cultures were discarded and 100 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] (0,5 mg/ml) containing PBS was added to each well. The cells were incubated for 2 to 4 hours at 37^oC. Finally the MTT crystals were dissolved by adding 100 µl of solubilization solution (81 v/v% isopropanol, 9 v/v% 1M HCl, 10 v/v% TritonX-100) and the formazan dye was measured in a microplate reader (FlexStation 3 Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) at 550 nm. All experiments were performed in triplicates.

Measurement of ATP

Media collected from primary macrophages were centrifuged and ATP content were determined from cell-free supernatants immediately after collection by ATPlite luminescence assay kit (PerkinElmer, Budapest, Hungary) according to the manufacturer's instructions and luminescence was measured by Wallac 1420 Victor2 fluorimeter-luminometer (Wallac Oy, Turku, Finland).

ELISA

Supernatants collected from MFs were centrifuged and stored at -20 ^{o}C until further use. IL-6, TNFα, IL-10 and IL-8 were detected from the ATP-untreated samples, and IL-1β was measured from ATP-untreated and -treated samples by ELISA (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Analysis was performed on a

FlexStation 3 Microplate Reader. The minimum detectable doses are 0.8 pg/ml for IL-1β and IL-8, 2 pg/ml for IL-10 and TNFα and 2.2 pg/ml for IL-6.

RNA preparation and RT-PCR

After removal of the supernatants by centrifugation, total RNA was isolated from the cell pellets by TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. The concentration and homogeneity of RNA was determined by a spectrophotometer (NanoDrop ND1000; Promega Biosciences, Madison, WI, USA). Standardized amounts of RNA were digested with DNase (Ambion , Austin, TX, USA) then reverse transcribed into cDNA by using SuperScript II First-strand Reverse Transcriptase and oligo dT primers (Thermo Fisher Scientific).

Quantitative real-time polymerase chain reaction (qPCR)

For gene expression analyses, PCR reactions of cDNA samples were measured using an ABI Step One Plus machine (Thermo Fisher Scientific). For the amplification reactions, all TaqMan Gene Expression Assays were purchased from Thermo Fisher Scientific. Taq DNA Polymerase (Fermentas, Vilnius, Lithuania) was used for amplification, and Rox Reference Dye (Thermo Fisher Scientific) was used for normalization of fluorescent reporter signal. Expression levels of target transcripts in each sample were calculated by the comparative Ct method after normalization to the human cyclophilin as control.

Western Blot analysis

Cells were harvested and centrifuged. From the supernatants, proteins were precipitated by 20% trichloroacetic acid (TCA). Pellet was washed in acetone and then dried, followed by re-suspensioned in loading buffer (62,5 mM Tris-HCl, pH 8.8, containing 25 % glycerol, 2 % SDS, 1 % β-mercaptoethanol and 1 % BPB). Cells were lysed in loading buffer. Before loading, all samples were boiled for 10 minutes. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked with 5% nonfat milk, washed briefly, thereafter incubated with primary antibodies (Abs) at 4°C overnight. Pro-IL-1β, procaspase-1, Akt1 and ASC Abs were from Santa Cruz Biotechnology (CA, USA), cleaved IL-1β (p17) and cleaved caspase-1 (p20) Abs were from Cell Signaling Technology (Danvers, MA, USA), NLRP3 Ab was from AdipoGen (San Diego, CA, USA), P2X7R Ab was from Alomone labs (Jerusalem, Israel), p-Akt (S473) was from R&D systems

(Minneapolis, MN, USA), p-ERK1/2 was from Sigma-Aldrich (St. Louis, MO, USA), p-IκBα, IκBα, ERK1/2, p-SAPK/JNK, SAPK/JNK, p-p38 and p38 MAP Kinase Abs were obtained from Cell Signaling Technology (Danvers, MA, USA). Primary Abs were used in 1:1000 dilution overnight then incubated with corresponding HRP-conjugated secondary Abs in 1:2000 dilution from Bio-Rad Laboratories (Hercules, CA, USA) for 1 hour at room temperature. Proteins were visualized by Supersignal West-Pico peroxide/luminol enhancer solution from Pierce Biotechnology (Rockford, IL, USA). To verify the loading of equal amount of protein sample, the β-actin (Sigma-Aldrich) expression was detected.

Measurement of caspase-1 activity

Caspase-1 activity in cell lysates was determined using the acetylated and AMC-conjugated fluorometric peptide substrate Acetyl-Tyr-Glu-Val-Asp-7-amino-4-methyl-coumarin (AnaSpec, San Jose, CA, USA). Lysis of the cells was performed on ice for 30 min in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5 mM EDTA, 0.5 % Nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin, 0.5 μg/ml pepstatin, 1.25 μg/ml leupeptin and 1 mM dithiothreitol. After centrifugation (10 000 g, 10 min at 4 ^{o}C), 30 μg protein lysate supernatant was incubated in 100 μl lysis buffer with 40 μM substrate (final concentration) in microtiter plate wells at room temperature, and the increase of fluorescence due to the release of AMC was detected at 460 nm, using 355 nm excitation wavelength in a Wallac 1420 Victor2 fluorimeter-luminometer (Wallac Oy, Turku, Finland).

siRNA transfection

On day 3 of differentiation, supernatants were collected and cells were suspended in PBS, then washed once with OptiMEM (Invitrogen, Carlsbad, CA, USA). Cells were transfected with NLRP3-specific siRNA (s38591, s38592, s38593) or with siRNA for negative control (Ambion) using an electroporator (Gene Pulser Xcell, Bio-Rad). Finally, cells were plated in 24-well tissue culture plates ($1x10^6$ cells / ml). On day 5, macrophages (M-MFs, GM-MFs) were stimulated with LPS (500 ng/ml).

Statistical analysis

Significant differences between mean values of two groups were evaluated using a Student's *t* test. Data presented as mean ±SD. One-way ANOVA, followed by Bonferroni post hoc test, was used for multiple comparisons.

4. Results

4.1. Different dynamics of NLRP3 inflammasome-mediated IL-1β production in GM-CSF- and M-CSF- differentiated human primary macrophages

4.1.1. LPS treatment of M-MFs and GM-MFs results in different pattern of cytokine expression

Our results show that both MF types produced detectable amount of TNFα and IL-8, however, the secretion kinetics and cumulative values showed significant differences. Although TNFα production by M-MF was moderate and reached maximum levels at an early time point (6h), TNFα secretion by GM-MFs was more robust and prolonged. The production of IL-8 was increased by both macrophages upon activation. Nevertheless, IL-6 was hardly detectable in the supernatant of M-MFs, meanwhile its production was more intense and prolonged by GM-MFs. In contrast, we observed that LPS treatment induced a continuously increasing IL-10 secretion by M-MFs, while GM-MFs were unable to produce detectable amount of IL-10.

Since several groups reported that IL-1β release by macrophages requires ATP supplementation, we collected media both from ATP-treated and non-treated cells. Surprisingly, following ATP exposure, the LPS-primed M-MFs released high amount of IL-1β at a very early time-point as 2 h, thereafter it decreased successively by time. In contrast, we found that IL-1β production by GM-MFs was gradually increased by time and its peak amount was comparable with that of the maximum of M-MFs. As we expected, we did not detect secreted IL-1β cytokine from the supernatant of LPS-treated M-MFs without triggering by ATP. However, to our surprise, LPS-primed GM-MFs secreted moderate, but significant amount of IL-1β even without ATP supplementation.

4.1.2. LPS-induced IL-1β production is mediated through caspase-1–dependent NLRP3 inflammasome pathway in both macrophage subtypes

To study the role of caspase-1 on IL-1β production, we used specific inhibitor of the enzyme, and monitored IL-1β secretion by MFs following LPS treatment. We found that the caspase-1 inhibitor Z-YVAD-FMK significantly reduced IL-1β production by both ATP-triggered macrophage subtypes. Moreover, the ELISA results also demonstrated that caspase-

1 enzyme is required for IL-1β release by LPS-primed GM-MFs in the absence of ATP treatment.

Previous studies have shown that reduction of intracellular K^+ concentration is sufficient to activate NLRP3 inflammasome. To clarify the type of inflammasome involved in LPS-primed IL-1β production, macrophages were exposed to LPS in the presence of high KCl concentration, which inhibits K^+ efflux. We observed a significantly suppressed IL-1β production by both macrophage types. Similar results were observed after knockdown of NLRP3 protein that confirms the necessity of NLRP3 inflammasome.

4.1.3. Extracellular ATP released by GM-MFs does not affect NLRP3 inflammasome activation directly

Previous studies reported that monocytes secrete IL-1β upon LPS challenge as a result of paracrine or autocrine effects of released endogenous ATP that triggers NLRP3 inflammasome activation. In contrast, macrophages are unable to process IL-1β upon LPS treatment due to the lack of ATP release. When we measured the extracellular ATP content of the supernatant, we found that untreated GM-MFs release ATP in a significantly higher concentration compared to that of M-MFs.

Extracellular ATP triggers NLRP3 inflammasome actiation through P2X7R. We found that LPS significantly induced the expression of the receptor in both MF types. However, we observed a faster decline in the expression in case of M-MFs, as compared to GM-MFs. We found that the inhibitor abolished ATP-triggered IL-1β secretion by both LPS-primed macrophage types. However, the pharmacological inhibition of P2X7 receptor or using apyrase enzyme that hydrolyzes extracellular ATP failed to reduce IL-1β secretion by LPS-primed GM-MFs without triggering with ATP. These data demonstrate that LPS-mediated IL-1β secretion in GM-MF is independent of P2X7 receptor.

4.1.4. CD39 and CD73 ectonucleotidases contribute to IL-1β secretion in GM-MFs

CD39 and CD73 can determine the concentration of ATP in the extracellular milieu, therefore we next studied the expression of these ectonucleotidases by both MF types. We found that although prolonged treatment of MFs with LPS resulted in induction of CD39 transcription, it was still significantly lower in GM-MFs compared to that of M-MFs. In contrast, while the transcription of CD73 was temporarily increased in M-MFs, it was markedly elevated in GM-MFs 24 h following LPS activation.

To see the effect of ectonucleotidases on endogenous ATP catabolism and IL-1β release of GM-MFs, we treated the cells with the ectonucleotidase inhibitor ARL67156. However, ATP content was significantly elevated in the presence of ARL67156, IL-1β secretion by LPS-primed GM-MFs was decreased. These results are implying the possibility that in GM-MFs not the endogenously released ATP, but the accumulating side- or end-product of ATP hydrolysis is required for IL-1β secretion.

4.1.5. Adenosine enhances IL-1β secretion in LPS-activated GM-MFs

To clarify the effect of adenosine on IL-1β production by M- and GM-MFs, first we measured the four adenosine receptor expression level upon LPS activation. Our qPCR results showed, that the expression of A2b and A3 was temporarily increased, whereas prolonged cultivation of LPS-primed macrophages significantly downregulates it. Meanwhile, transcription of A2a was significantly induced and only moderately decreased in GM-MFs in contrast to M-MFs, where the A2a receptor was dramatically decreased. In most time points A2a and A2b receptors are expressed at a higher level in GM-MFs compared to M-MFs. Interestingly, A1 receptor was hardly detectable in both macrophages.

When we used adenosine receptor specific antagonists, we found that A2a and A2b specific antagonists significantly decrease the LPS-mediated IL-1β secretion, while A1 and A3 specific antagonists did not cause significant changes. Furthermore, when we treated GM-MFs with an increasing amount of adenosine, we detected enhanced LPS-mediated IL-1β secretion. Similar results were obtained for GM-MFs in the presence of ATP supplementation. Interestingly adenosine had no effect on IL-1β secretion by LPS-primed M-MFs. These results indicate that adenosine has an important regulatory effect solely on IL-1β secretion by GM-MFs, independently of ATP supplementation, while it does not affect IL-1β secretion by M-MFs.

4.1.6. Differences in expression of NLRP3 and pro-IL-1β as well as in caspase-1 enzyme activity mediate diversity in IL-1β processing by GM- and M-MFs

To further study the molecular basis of the observed differences in IL-1β production by M-MFs and GM-MFs, we stimulated cells with LPS, and assessed NF-κB and MAPK signaling. We found that ERK1/2 protein was already phosphorylated in the non-treated M-MFs and it was further induced by LPS for up to 30 min. In LPS-primed GM-MFs the activation of ERK1/2 was observable by 30min then it was down-regulated. We detected

similar kinetic in the activation of p38 and SAPK/JNK in the two MF types, however, the intensity was stronger in M-MFs. Interestingly, while LPS treatment resulted in a strong phosphorylation of IκBα at 10 min in the GM-MFs, in the case of M-MFs, it showed a shift (60 mins).

Nevertheless, we detected significant differences in the expression of the NLRP3 sensor molecule and pro-IL-1β. NLRP3 was clearly detectable in non-activated M-MFs, and enhanced by LPS treatment peaked by 2 h, and decreased gradually at later time points. In contrast, in untreated GM-MFs, expression of NLRP3 was hardly detectable, but LPS treatment resulted in a significant and continuously increasing NLRP3 protein expression. We found that untreated macrophages did not produce pro-IL-1β, though LPS priming strongly and rapidly induced its expression in both MFs. However pro-IL-1β was strongly detected by 2 h following LPS treatment in M-MFs, declined rapidly by 24 h, GM-MFs expressed this protein continuously.

We detected intensive caspase-1 band following brief LPS treatment (2 h) in M-MFs, which was abolished by 12 h. In the case of GM-MFs, the active form of caspase-1was weakly detectable, however, it showed an increasing tendency with time. Therefore, we determined also the caspase-1 activity. We found that it was hardly detectable in the absence of ATP in LPS-primed M-MFs, although it was significantly induced up to 2 h by triggering with ATP. Surprisingly, enzyme activity was clearly detectable in LPS-primed GM-MFs without triggering with ATP, even in the absence of LPS. Additionally, ATP enhanced caspase-1 activity at most of time points in the GM-MFs.

4.1.7. Rapid attenuation of IL-1β secretion by M-MFs is the result of the robust secretion of IL-10

We detected a strongly increasing IL-10 expression by M-MFs in parallel with the decrease of IL-1β. We aimed to investigate the role of released IL-10 in rapid down-regulation of IL-1β secretion detected in M-MFs. We found that secretion of IL-1β was significantly reduced by the administration of rhIL-10 cytokine. Furthermore, by blocking IL-10 binding to their receptor, we demonstrated significantly elevated IL-1β secretion as well as transcription by M-MFs.

One of the most important signaling pathways affected by IL-10 is the Akt signal transduction cascades, which is inhibited by IL-10 receptor ligation. We showed that Akt phosphorylation was significantly weaker in M-MFs at each time points following LPS stimulation compared to GM-MFs. When M-MFs were co-treated with LPS and rhIL-10, we

observed a significantly reduced Akt phosphorylation compared to that induced by LPS alone, indicating that IL-10 indeed affects this pathway in M-MFs.

4.2. *Aloe vera* downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages

The anti-inflammatory activity of *Aloe vera* has been evaluated in a number of inflammation models partly *via* down-regulation of pro-inflammatory cytokine production such as IL-1β. We demonstrated the mechanism that likely contributes to the reduced IL-1β production by activated human macrophages treated with *Aloe vera*.

4.2.1. GM-MFs are substantially more sensitive for *Aloe* treatment than THP-1 macrophages

First, we determine the cytotoxic effect of *Aloe vera* on human macrophages. We found that using *Aloe vera* up to 10 v/v % did not significantly affect the viability of THP-1 cells. Interestingly, 5 v/v % of *Aloe vera* already resulted in more than 50% cell death of GM-MFs, and 10 v/v % of *Aloe vera* led to more than 70% death of these cells.

4.2.2. *Aloe vera* significantly decreases cytokine production of LPS-activated macrophages

We determined, that *Aloe vera* itself exerted a non-significant effect on the production of all the measured pro-inflammatory cytokines in THP-1 cells, and had no effect on the primary macrophages. However, *Aloe vera* significantly reduced the LPS-induced secretion of IL-8, TNFα, IL-6 and IL-1β cytokines in both cell types in a concentration-dependent manner. Furthermore, our results also demonstrated that while in the case of GM-MFs *Aloe vera* (3 v/v %) resulted in more than 70 % reduction in cytokine production, in contrast the attenuation of cytokine production was less pronounced in THP-1 cells, even with the 10 v/v % of *Aloe vera* treatment.

4.2.3. *Aloe vera* attenuates the LPS-induced expression of IL-1β

GM-MFs were treated with 3 v/v % of *Aloe vera* in the presence or absence of LPS. Our Results showed that *Aloe vera* treatment significantly decreased the protein expression of

both pro-IL-1β and cleaved IL-1β forms in LPS-activated GM-MFs. Moreover, we determined that *Aloe vera* reduced LPS-induced pro-IL-1β expression at transcriptional level in a dose-dependent manner.

4.2.4. *Aloe vera* attenuates LPS-induced expression of NLRP3 and caspase-1 in GM-MFs

To see whether expression of NLRP3 inflammasome components are affected by *Aloe vera,* GM-MFs were activated with LPS in the presence or absence of *Aloe vera,* and the transcription as well as protein expression of NLRP3 and procaspase-1 was studied. *Aloe vera* treatment alone did not affect the transcription and the expression of the studied inflammasome components. However, it substantially down-regulated their LPS-induced transcription, and this effect was also detectable at the protein level.

4.2.5. *Aloe vera* down-regulates the LPS-induced expression of P2X7 receptor

Previously, we assigned that P2X7 receptor is expressed and responsible for IL-1β release triggered by ATP treatment in LPS-primed GM-MFs. Next, we aimed to study whether *Aloe vera* would decrease IL-1β secretion *via* influencing the expression of P2X7R. We found that LPS-induced expression of P2X7 receptor was abolished by *Aloe vera* treatment. These results implicate the involvement of depleted P2X7 receptor store in reduced IL-1β secretion, while revealing the impact of *Aloe vera* on upstream mechanisms,

4.2.6. *Aloe vera* inhibits the LPS-induced activation of NF-κB, p38, JNK and ERK signal transduction pathways

To explore whether *Aloe vera* has an effect on LPS-induced activation of signal transductions, macrophages were treated with LPS in the presence or absence of *Aloe vera* and the time-dependent phosphorylation of key signaling molecules were followed by Western blot. Erstwhile we described that LPS induces strongly the phosphorylation of IkBα in GM-MFs. Interestingly, it was completely abolished by *Aloe vera* treatment. Unlike NF-kB pathway, the LPS-induced phosphorylation of p38, ERK 1/2 and SAPK/JNK were also prevented by *Aloe vera* treatment. Altogether, these results show that *Aloe vera* treatment results in a broad spectrum of inhibition of signal transduction pathways.

5. Discussion

IL-1β is a pivotal cytokine that regulates many immunologic and physiologic functions and due to its important role, the secretion of this cytokine is tightly regulated by the activation of caspase-1 occurs *via* recruitment to the NLRP3 inflammasome. IL-1β is produced and secreted by a variety of cell types such as macrophages which are very plastic cell population Therefore a huge spectrum of protocols were developed to enable the study of different types of MF in *in vitro* conditions. We reported for the first time a comparative study about the dynamics and molecular mechanisms of NLRP3 inflammasome activation, in the generally accepted two distinct macrophage subtypes differentiated by M-CSF (M-MFs) and GM-CSF (GM-MFs), respectively. We focused on the LPS-induced IL-1β secretion by these human macrophages, using ATP supplementation, which is a triggering DAMP for NLRP3 inflammasome activation.

First we compared the cytokine profile of these macrophages upon LPS stimulation and found that GM-MFs display enhanced production of pro-inflammatory cytokine, more markedly compared to that of M-MFs. Furthermore only M-MFs were capable of secreting IL-10 anti-inflammatory cytokine. These results are in good agreement with the general notion that human macrophages generated from monocytes in the presence of GM-CSF predominantly have a common pro-inflammatory phenotype that leads to perpetuation of inflammation. Contrarily, M-CSF treatment induces human monocytes to adopt potent anti-inflammatory macrophage phenotype that dampen pro-inflammatory cytokines and may be essential for tissue repair and wound healing.

Surprisingly, we found that in the presence of ATP both macrophage subtypes produced comparable amount of IL-1β, although at very different time kinetics. While secretion by M-MFs was a very rapid process and lasted only for a few hours after LPS treatment, in case of GM-MFs it was a slower process that led to gradually increasing IL-1β secretion. It is commonly accepted that macrophages require two signals for NLRP3 inflammasome activation. The priming signal ensures the expression of required components, while the second signal, like ATP, triggers the assembly and activation of the complex. Several groups reported that IL-1β release by macrophages requires both signal. As we expected, in the absence of a second stimulus, we did not detect IL-1β from the supernatant of the LPS-primed M-MFs, however, surprisingly, under similar conditions, we detected IL-1β production of GM-MFs, in an amount comparable to that produced by monocytes.

ATP is released from cells into the extracellular space in response to inflammation or tissue injury. Previous studies reported that human monocytes stimulated with microbial components release ATP in an autocrine / paracrine manner, which is responsible for the purinergic P2X7 receptor-dependent activation of NLRP3 inflammasome and leads to IL-1β secretion. Our results demonstrated that both macrophage subtypes released nanomolar ATP, however, it was significantly higher in the culturing medium of GM-MFs compared to that of M-MFs. We also observed that both MFs expressed comparable amounts of functional P2X7 receptor. However, the P2X7R inhibitor failed to prevent IL-1β secretion by LPS-primed GM-MFs, nor apyrase treatment decreased the cytokine production by these cells, indicating that the LPS-induced IL-1β production by GM-MFs is independent of the released endogenous ATP. Based on our observations and published reports from the field, it seems that the low sensitivity of P2X7 receptors to ATP in both M-MFs and GM-MFs limits their activation to conditions in which immune cells or dying cells release millimolar concentration of ATP into the extracellular space. This threshold enables P2X7R to function as a danger sensor associated with inflammation or cell injury.

Following the release of ATP into the extracellular space, CD39 converts ATP into AMP, and then CD73 dephosphorylates AMP into adenosine thereby regulating immunity and inflammation. Our results showed that both MFs express CD39 and CD73 followed by LPS treatment, but with significant differences. We demonstrated that the LPS-induced increase of CD39 expression was significantly higher in M-MFs than in GM-MFs. In contrast, expression of CD73 that induces extracellular accumulation of adenosine resulted in a much more robust expression in GM-MFs compared to M-MFs. The ability of these macrophage subtypes to express different ectonucleotidases led us to speculate that the level of by- and end-products of ATP hydrolysis, such as adenosine, may have an important indirect regulatory effect on IL-1β production by GM-MFs. This notion is supported by our results showing a significant decrease in IL-1β secretion solely upon LPS activation in GM-MFs in the presence of ectonucleotidase inhibitor, while the extracellular ATP level was significantly increased.

Adenosine was reported to be a key regulator of IL-1β secretion, and our results show that in the absence of ATP supplementation, adenosine treatment increased the LPS-induced IL-1β secretion by GM-MFs in a dose dependent manner. We also found that IL-1β secretion by LPS primed GM-MFs was significantly reduced by A2a or A2b specific receptor inhibitors, which receptor's expression is more significant in the GM-MFs than in the M-MFs. A2a and A2b receptor activation stimulate the cyclic AMP formation, which can bind to and

modulate the function of nonselective cation channels that conduct calcium and potassium fluxes. Both are important triggers of NLRP3 inflammasome activation.

Furthermore, we found that adenosine can enhance LPS-induced IL-1β secretion in the presence of ATP only in GM-MFs. Importantly we did not notice similar effect in the case of M-MFs. These findings support that the distribution of adenosine receptors appear critical in the regulation of inflammation.

Next we examined the role of NLRP3 inflammasome in the control of IL-1β secretion by both macrophage subtypes, for the reason of various inflammasome complexes being associated with IL-1β production. Using caspase-1 specific inhibitor or silencing of NLRP3 protein, have confirmed that IL-1β secretion by M-MFs and GM-MFs, respectively requires the canonical caspase-1 – dependent NLRP3 inflammasome function. When we compared the expression profile of the inflammasome components between these cells, we observed vast differences in the expression dynamics of NLRP3 as well as pro-IL-1β, as in M-MFs, LPS treatment led to a rapid increase of these proteins, but only for a short time interval, while the same treatment induced a slow, gradually increasing expression in GM-MFs. Our results also demonstrated the requirement of the priming step for the pro-IL-1β expression in both types of macrophages, however a striking difference was found in the expression of NLRP3 between these cells that was constitutive expressed in M-MFs. We speculated different molecular mechanisms underlying the observed differences in the expression of these proteins in the two cell types. Therefore we analyzed various signaling pathways and our results suggest that in the early events of priming in M-MFs is involved the rapid and pronounced activation of MAPK signaling after LPS treatment. Furthermore we detected strong ERK phosphorylation in M-MFs even in non-treated cells. We conclude that the constitutive baseline activation of ERK resulted in a "half-primed" condition of M-MFs and also provides continuous basal expression of NLRP3, which is normally provided by LPS in GM-MFs.

To investigate the differences in activation of NLRP3 inflammasome between these cell types, we analyzed the function of caspase-1 enzyme, which was shown above to play essential role in IL-1β secretion by both macrophage subtypes. Our findings demonstrated that besides priming, that is required for both macrophage types for *de novo* synthesis of proIL-1β and upregulation of NLRP3, a second signal which can be provided by ATP is absolutely necessary for the activation of caspase-1 in M-MFs, but not in GM-MFs. Furthermore, we assumed that the observed IL-1β release by GM-MFs in the absence of ATP supplementation is due to the constitutively active enzyme.

We have found another interesting correlation as LPS treatment strongly induces increasing IL-10 secretion by M-MFs, in parallel with the time-dependent decrease of IL-1β release. By inhibiting IL-10 binding to IL-10R, we demonstrated that the observed decrease in IL-1β production by M-MFs is, at least in part, mediated by IL-10. Furthermore, we detected a significant weaker activation in Akt signaling pathway in M-MFs compared to GM-MFs following LPS treatment, which is one of the most important signaling pathways affected by IL-10. Based on reports, IL-10 can abolish Akt phosphorylation that in turn leads to the decreased phosphorylation of downstream signaling pathways. These findings and our results may explain the down-regulation of JNK and ERK phosphorylation following LPS stimuli in M-MFs. Considering that these pathways have an important regulatory role in the priming process of NLRP3 inflammasome, the observed down-regulation of NLRP3, pro-IL-1β expression and the following attenuation of IL-1β secretion in M-MFs, at least partly, may be explained by the indirect inhibitory effect of IL-10.

Our results may widen the general knowledge about macrophages, a heterogeneous population with diverse phenotypes that facilitate effective immune responses to the ever-changing environment. A high production of pro-inflammatory mediators have been closely related with a deficient activation of macrophages as well as the pathogenesis of several chronic diseases with inflammatory basis; such as rheumatoid arthritis, atherosclerosis, diabetes and cancer. Therefore, further insight into IL-1β production of macrophages would be of great benefit to clinical immunology, providing an overview of potential targets.

Aloe vera is a high-quality wound care product that has been used traditionally for centuries by different cultures. In addition, the active components lead to broad health benefits; such as anti-diabetic, anti-cancer and antimicrobial. The anti-inflammatory activity of *Aloe vera* has been intensively studied over the past few decades; however detailed molecular mechanism of its effect is only being explored in recent years. In our work we examined the effect of *Aloe vera* gel supernatant on the IL-1β production of the earlier well defined inflammatory macrophage type, GM-MF and the human macrophage-like THP-1 cell line. Our results show that *Aloe vera* treatment significantly down-regulated LPS-induced IL-8, TNFα, IL-6 and IL-1β inflammatory cytokine production in a concentration dependent manner in both THP-1 cells and GM-MFs. These results are in line with studies demonstrating anti-inflammatory effects of *Aloe vera* in animal models without molecular explanation of the effect on IL-1β and TNFα production in non-differentiated THP-1 cells. We found that not only the cytotoxic sensitivity of primary GM-MFs was higher than that of THP-1 cells, but also the susceptibility towards *Aloe*-mediated down-regulation was more

pronounced in these cells. To obtain results close to *in vivo* status, our further studies on the molecular characterization of the *Aloe*'s effects on IL-1β production focused on primary macrophages. Our results demonstrate that the LPS-induced caspase-1 and NLRP3 expression was significantly decreased in the presence of *Aloe vera* solution both at mRNA and protein levels. Moreover, we also determined a down-regulatory effect of *Aloe vera* on the pro-IL-1β expression in LPS-activated GM-MFs that limits the function of activated inflammasome complexes as their primary substrate. As our previous results showed the ATP-triggered NLRP3 inflammasome-mediated IL-1β production by LPS-primed GM-MFs was P2X7 receptor dependent mechanism. Accordingly, we studied the effect of *Aloe vera* on the P2X7 receptor expression and we found that it completely abolished the effect of LPS induction on the expression of this ATP-sensor.

Furthermore, our results showed that the activation of LPS induced signaling pathways were significantly inhibited by *Aloe vera* treatment. These results suggest that *Aloe vera* inhibits IL-1β production by GM-MFs at the level of priming step, upstream of the NLRP3 inflammasome. However, the observed changes in the expression of the ATP-sensor P2X7 receptor may also contribute to the pronounced reduction of IL-1β secretion, underlying a versatile and efficient anti-inflammatory effect of *Aloe vera*. Thereby, *Aloe vera* could be a new therapeutic tool to target NLRP3 inflammasome-mediated cytokine production.

Our results may help clarify some controversial reports within the NLRP3 inflammasome field; the divergences of which are derived from methodological, cell type, and species differences in studies of NLRP3 inflammasome regulation.

6. Summary

Macrophage phenotype and function are dependent and driven by the complex and frequently changing cellular and molecular microenvironment *in vivo*. In human system, *in vitro* M-CSF and GM-CSF are the most frequently used to differentiate blood monocyte-derived M-MFs or GM-MFs, respectively. The general notion that GM-CSF treatment of monocytes results in the formation of macrophages with enhanced bactericidal capacity and secretion of pro-inflammatory mediators stands. In contrast, M-CSF treatment leads to the generation of macrophages with a decreased ability to produce inflammatory cytokines and participate in wound healing and tissue regeneration.

When we compared LPS-induced cytokine secretion by these cells, we found that pro-inflammatory cytokine (TNFα, IL-6 and IL-8) production was more pronounced in GM-MFs compared to that of M-MFs, while only M-MFs produced IL-10 anti-inflammatory cytokine. Surprisingly, our results showed that in the presence of the second signal, ATP, both LPS-primed MF subtypes produced comparable amount of IL-1β, albeit with very different dynamics. IL-1β secretion by LPS-primed M-MFs was a very rapid and short event, and is attenuated in part by the presence of IL-10 which reduces Akt signaling. However, IL-1β production by GM-MFs develops gradually, and these cells were capable of producing moderate amounts of IL-1β even in the absence of ATP supplementation, due to the constitutively active caspase-1 enzyme. The observed IL-1β release was independent of the P2X7 receptor signaling, and was not directly influenced by the released endogenous ATP by GM-MFs. The actual amount of extracellular ATP is determined by the balance of ATP released from cells and ATP hydrolyzed by the membrane-bound ectonucleotidases. We have shown that the ectonucleotidases CD39 and CD73 were differentially expressed in these macrophages, and they played an important regulatory role in the IL-1β secretion in GM-MFs. Furthermore, we provided evidences that adenosine; one of the end-products of ectonucleotidase function, enhanced LPS-primed IL-1β secretion by GM-MFs, this stimulatory effect however was not observed in M-MFs. Our results showed that due to the different activation status and expression levels of the NLRP3 inflammasome components, as well as the diverse signaling activity of the pathways, the two subtypes of macrophages responded very differently to the same stimuli.

To study the anti-inflammatory *Aloe Vera* on IL-1β secretion by human macrophages, we chose GM-MFs with prolonged pro-inflammatory properties. We showed that *Aloe vera*-mediated strong reduction of IL-1β appeared to be the consequence of the reduced expression

of both pro-IL-1β as well as NLRP3 inflammasome components, through suppression of specific signal transduction pathways. Furthermore, we demonstrated that expression of the ATP sensor P2X7 receptor was also downregulated by *Aloe vera,* which may have also contribute to the attenuated IL-1β cytokine secretion.

Intensive studies have advanced our knowledge about the general mechanisms of NLRP3 inflammasome function and its regulators. We believe that our results shed light on some controversial results in the field of the NLRP3 inflammasome, and also clarify that the actual outcome regarding the activation and and production of IL-1β is strongly determined by the molecular characteristics of the cell type, involving various intracellular or extracellular modulators. Moreover, our results may provide a potential therapeutic approach, to better manage IL-1β- mediated systemic or chronic diseases.

7. Publication list prepared by the Kenézy Life Sciences Library

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Registry number: DEENK/73/2017.PL
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Candidate: Marietta Margit Budai
Neptun ID: RYGTX2
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List of publications related to the dissertation

1. **Budai, M. M.**, Tőzsér, J., Benkő, S.: Different dynamics of NLRP3 inflammasome-mediated IL-1[béta] production in GM-CSF- and M-CSF-differentiated human macrophages.
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2. **Budai, M. M.**, Varga, A., Milesz, S., Tőzsér, J., Benkő, S.: Aloe vera downregulates LPS-induced inflammatory cytokine production and expression of NLRP3 inflammasome in human macrophages.
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Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

List of other publications

3. Varga, A., **Budai, M. M.**, Milesz, S., Bácsi, A., Tőzsér, J., Benkő, S.: Ragweed pollen extract intensifies LPS-induced priming of NLRP3 inflammasome in human macrophages. *Immunology. 138* (4), 392-401, 2013.
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Address: 1 Egyetem tér, Debrecen 4032, Hungary Postal address: Pf. 39. Debrecen 4010, Hungary
Tel.: +36 52 410 443 Fax: +36 52 512 900/63847 E-mail: publikaciok@lib.unideb.hu, ¤ Web: www.lib.unideb.hu

Oral presentations

Budai MM.: Immunomodulatory effect of *Aloe vera* extract on human macrophages
5th Molecular Cell and Immune Biology Winter Symposium, 2012, Galyatető, Hungary

Budai MM.: Possible molecular differences in the mechanisms of IL-1β production by human monocyte-derived different macrophages
6th Molecular Cell and Immune Biology Winter Symposium, 2013, Galyatető, Hungary

Budai MM., Varga A., Benkő S. : Lipopolysaccharide induces IL-1β production via P2X7 receptor – independent mechanisms in human GM-MFs
Annual Meeting of the Hungarian Biochemical Society, 2014, Debrecen, Hungary

Budai MM., Becsei A., Varga A., Tőzsér J., Benkő S. : Molecular mechanisms of IL-1β production and NLRP3 inflammasome function in LPS-activated human macrophage subtypes
Annual Meeting of the Hungarian Biochemical Society, 2014, Debrecen, Hungary

Poster presentations

Budai MM., Varga A., Tőzsér J., Benkő S.: NLRP3 inflammasome - mediated IL-1β secretion by diverse macrophage types primed with LPS
76th Anual Meeting of The Hungarian Society for Physiology, 2012, Debrecen, Hungary

Budai MM., Varga A., Danis J., Tőzsér J., Benkő S. : NLRP3 inflammasome mediated IL-1β production by LPS in different phenotypes of macrophages
European Macrophage and Dendritic Cell Society Meeting, 2012, Debrecen, Hungary

Budai MM. Danis J., Varga A., Csernoch L., Tőzsér J., Benkő S. : Molecular differences in the mechanisms of NLRP3 inflammasome-mediated IL-1β production by LPS-activated human monocyte-derived macrophage subpopulations
8th ENII –Summer School, 2013, Sardinia, Italy

Budai MM., Danis J., Varga A., Varga D., Csernoch L., Tőzsér J., Benkő S. : Mechanisms behind NLRP3 inflammasome–mediated IL-1β production by LPS-primed human macrophages
77th Anual Meeting of The Hungarian Society for Physiology, 2013, Budapest, Hungary

Budai MM., Danis J., Varga A., Csernoch L., Tőzsér J., Benkő S. : NLRP3 inflammasome-mediated IL-1β production by LPS in different phenotypes of human macrophages
42nd Annual Meeting of The Hungarian Society for Immunology, 2013, Pécs, Hungary

Budai MM.: Molecular mechaninsms behind the diverse activation of NLRP3 inflammasome and IL-1β production by different phenotypes of human macrophages
'Debrecen for the life science research' organized by National Excellent Program, 2014, Debrecen, Hungary

Budai MM., Varga A., Csernoch L., Benkő S. : The lipopolysaccharide-induced NLRP3-dependent IL-1β production by GM-MFs is independent of the purinergic P2X7 receptor
44th Annual Meeting of the German Society for Immunology, 2014, Bonn, Germany

8. Keywords

Nod-like receptors, NLRP3 inflammasome, IL-1β cytokine, macrophages, P2X7 receptor, ATP, adenosine, signal transduction, *Aloe vera*

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